A KINETIC ANALYSIS OF THE ASSEMBLY OF MICROTUBULES IN VITRO

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1. Introduction

Microtubules have many different biological functions in which their dynamic properties play a central role. A detailed knowledge of the kinetics of assembly is therefore necessary for the understanding of their mechanism of action.

In this study the kinetics of polymerisation, as followed by turbidity measurements, is analysed. As previously shown, [1,2] two steps have to be considered: the nucleation and the propagation step. The turbidity measurements are correlated with electron microscopy which allows a quantitative analysis of the propagation kinetics. Our results correspond well with the data of Bryan [3] obtained with sonicated fragments. Information about the process of nucleation is also obtained. The role of the ring-oligomers, present in these preparations, is discussed both in the process of nucleation and propagation.

2. Materials and methods

Tubulin is isolated from pig brains, in the first polymerisation cycle according to the method of Shelanski [4]. The two subsequent cycles, and the polymerisation studies are done in MES-buffer of pH 6.4 (50 mM MES, 70 mM KCl, 1.0 mM GTP, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM NaN_3). The protein is stored without glycerol in liquid nitrogen. Protein concentration is determined by the method of Lowry [5], using bovine serum albumine (Serva) as standard. (S-)Tubulin prepared in this way, contains about 10% of microtubule-associated-proteins (MAPS) which induce the formation of ringlike oligomers at 4°C. Pure (PC-)tubulin protomers are prepared by phosphocellulose chromatography [6]. A mixture of protomers and MAPS is obtained by dissociation of the rings at high ionic strength (0.8 M KCl) and dilution back to 0.1 [7]. The same results were obtained by reaction with diamide at 3 mM, followed by the addition of excess dithiothreitol [8]. This mixture reassociates slowly. The ratio MAPS/tubulin is decreased by the addition of PC-tubulin.

For electron microscopy, solutions are diluted to 0.05 mg/ml with polymerisation buffer containing 50% ethylene glycol to prevent dissociation. The solutions are then applied to grids and stained with uranyl acetate.

Polymerisation is initiated by large temperature jumps from 4–30°C, in a cell constructed in the laboratory. Temperature changes exponentially with a half-life of 1.2 sec. The polymerisation reaction is followed by turbidity measurements at 350 nm. Turbidity was shown to be a measure of the weight concentration of the polymer [1].

3. Results and discussion

3.1. *Kinetics of propagation*

The time course of turbidity appearance upon polymerisation is sigmoidal, as shown in fig.1. The initial region of acceleration is not studied, because turbidity is not yet a measure of the weight concentration of the small polymers formed. Our analysis is confined to the linear phase and the approach to equilibrium.

Electron microscopy [9,10] reveals an extensive polymorphism of polymerised tubulin. Under our conditions of fast polymerisation, short microtubules are rapidly formed and rings disappear concomitantly. The approach to equilibrium is therefore attributed to the process of propagation or association of protomers to growing microtubules. In that case the rate of disappearance of protomers can be described by the model of Oosawa [11]:

$$-dc_1/dt = (k_+c_1 - k_-)m = k_+m(c_1 - c_c)$$

Here c_1 is the protomer concentration, k_+ and k_- are the on and off rate constants of propagation, m is the concentration of growing sites, assumed to be equal to the number concentration of microtubules, and c_c is the protomer concentration at equilibrium. With m constant, a pseudo first order process will be found with $k_{obs} = k_+m$. The logarithmic plot of fig.1 shows that the approach to equilibrium is indeed governed by first order kinetics. The relation between k_{obs} and m is obtained by studying the propagation kinetics of preformed microtubules. In these experiments a stock solution of microtubules is diluted with a solution of S-tubulin. Figure 2 shows that k_{obs} is proportional to the original concentration of microtubules multiplied with the dilution factor (f). Figure 2 also shows that the amplitude of the propagation reaction is proportional to the concentration of the added tubulin. The value of k_+ can now be calculated if m is known. This is obtained from the relation $m = c_p/\langle i \rangle$ [11]. Here c_p is the weight concentration of microtubules, determined from the total turbidity and the specific turbi-

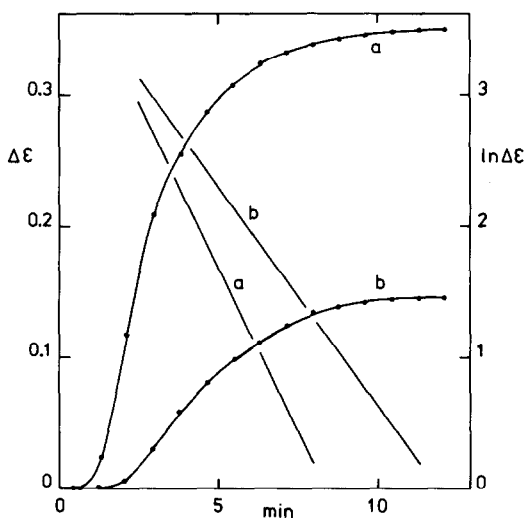

Fig.1. The appearance of turbidity (•-•-•) at 350 nm, upon the polymerisation of S-tubulin. Polymerisation is induced by a temperature jump from 4 to 30°C. (•-•-•) Turbidity and (———) logarithmic plot of the approach to equilibrium. (a) 2.9 mg/ml tubulin and k_{obs} = 0.37 min^{-1}; (b) 1.9 mg/ml tubulin and k_{obs} = 0.14 min^{-1}.

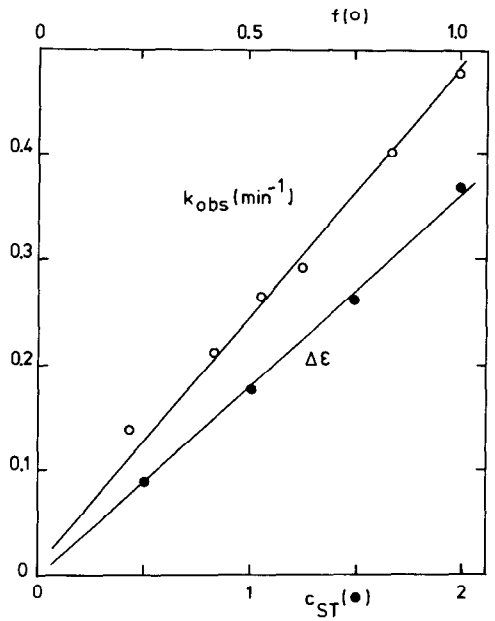

Fig.2. Propagation of preformed microtubules. Decreasing amounts of preformed microtubules are propagated by the addition of S-tubulin. The observed first order rate constant k_{obs} (○) is shown to be proportional to the original concentration of microtubules multiplied with the dilution factor f. At a constant amount of preformed microtubules, the amplitude (•) of the propagation reaction is proportional to the amount of added S-tubulin.

Table 1
Calculated rate constants of propagation

T	c_{tot}	k_{obs}	N	$\langle i \rangle$	k_+	k_-
25	2.5	0.20	125	12	2.7	22
	3.7	0.32	472	11	3.2	26
	3.8	0.20	115	12	2.2	18
	4.7	0.48	440	8	2.8	22
	5.7	0.55	832	6	2.0	16
30	3.8	0.58	330	8	4.8	30
	5.7	1.06	711	6	3.9	25
35	1.9	0.34	284	9	7.0	38
	3.1	1.00	393	6	6.7	36
	3.8	0.85	541	6	4.5	24

T, temperature in °C; c_{tot}, tubulin concentration in mg/ml; k_{obs}, observed rate constant of propagation in min^{-1}; N, number of microtubules counted; $\langle i \rangle$, average length in μm (rounded); k_+, on rate constant of propagation of S-tubulin in $10^6 M^{-1}$ sec^{-1}; k_-, off rate constant of propagation in sec^{-1}.

dity increase of 0.2 O.D. units/mg polymer at 350 nm. $\langle i \rangle$ is the average length determined by electron microscopy. The results of a series of experiments are shown in table 1. Typical length distributions obtained, are shown in fig.3. The data of table 1 correspond well with the results of Bryan [3] who used short microtubules obtained by sonication.

Although a mixture of protomers and rings was added to the preformed microtubules, a single first order process was observed in the propagation experiments of fig.2. This can be explained by a rapid interconversion between rings and protomers. The following considerations point to a dissociation of rings prior to propagation:

(i) electron microscopy shows that rings disappear rapidly upon polymerisation;
(ii) the same first order process is found when microtubules are allowed to grow with predissociated rings;
(iii) the reassociation of rings is relatively slow, as inferred from their much slower overall rate of polymerisation.

The dissociation of the rings is presumably due to the complexation of the MAPS by the microtubules present. The process is analogous to the rapid dissociation of rings by the addition of polyanions which were shown to complex the MAPS [3].

So far the MAPS/tubulin ratio was kept constant. This ratio can be decreased by the addition of PC-tubulin. When a mixture of S- and PC-tubulin is added to preformed microtubules, both k_{obs} and the amplitude of the reaction are lowered (not shown). The influence on the amplitude shows that MAPS decrease k_-, while the effect on k_{obs} could be a decrease of

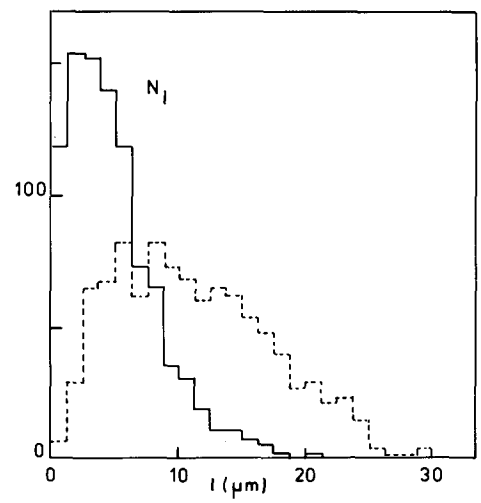

Fig.3. Typical length distributions obtained by polymerising tubulin at 25°C at 5.7 mg/ml (———) and at 3.7 mg/ml (– – –). Both distributions are normalized to a total of 1000 microtubules.

k_+ or a modulation of the concentration of active ends (m). A quantitative analysis of these effects has to wait for the purification and full characterisation of the MAPS.

3.2. *Nucleation*

Information about the process of nucleation can be obtained from the dependence of the number concentration of microtubules formed, on the initial tubulin concentration. This dependence can be found by plotting k_{obs} vs. the total tubulin concentration, provided the ratio MAPS/tubulin is constant.

When S-tubulin is polymerised at increasing concentrations, k_{obs} increases linearly (fig.4). This indicates that nucleation is favoured at higher concentrations. The length distributions of fig.3 confirm this, as it is clear that at higher concentration shorter microtubules are formed.

When however, a constant amount of S-tubulin is mixed with increasing amounts of predissociated rings and polymerised, k_{obs} does not increase, while the amplitude of the polymerisation reaction increases normally, as shown in fig.4.

The same type of experiment can be done with PC-tubulin. The same results are obtained, except that the amplitude increases with a smaller specific turbidity, as expected from the smaller MAPS concentration.

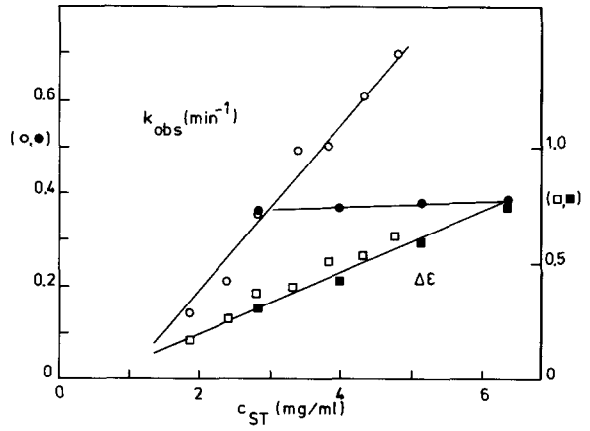

Fig.4. Polymerisation of S-tubulin at increasing initial tubulin concentration. Temperature is raised from 4–30°C. From a critical concentration on, k_{obs} (○) and the amplitude (□) increase linearly with tubulin concentration. When however, protomers (+ MAPS) are added to a constant amount of S-tubulin, k_{obs} (●) remains constant, while the total amplitude (■) increases normally. In this case the weight concentration of the polymer increases while the number concentration remains constant.

These results lead to the following conclusions:
(i) Both states of tubulin contribute to propagation;
(ii) only S-tubulin contributes to nucleation.

Our interpretation is that nucleation starts by the association of rings (probably broken open). The added protomers do not contribute to nucleation, probably because ring formation is slower and becomes more and more defavourized because both protomers and MAPS associate with the nuclei and microtubules formed.

Although rings initiate the formation of microtubules in these conditions, they are not necessarily intermediates when polymerisation is started from pure protomers. These can be induced to polymerise by the addition of glycerol, DMSO, MAPS or polycations at temperatures higher than 4°C. In these cases rings are never seen in electron microscopy [3]. It is therefore probable that shorter intermediary filaments are already able to grow in the lateral direction. At 4°C, however, lateral interactions are unstable and the longitudinal associations proceed until long filaments are formed that curl into rings.

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References

[1] Gaskin, F., Cantor, C. R. and Shelanski, M. L. (1974) J. Mol. Biol. 89, 737–758.
[2] Engelborghs, Y., Heremans, K., De Maeyer, L. C. M. and Hoebeke, J. (1976) Nature 259, 686–689.
[3] Bryan, J. (1976) J. Cell Biol. 71, 749–767.
[4] Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.

[6] Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–1862.
[7] Kirschner, M. W., Williams, R. C., Weingarten, M. and Gerhart, J. C. (1974) Proc. Natl. Acad. Sci. USA 71, 1159–1163.
[8] Mellon, M. G. and Rebhun, L. I. (1976) J. Cell Biol. 70, 226–238.
[9] Erickson, H. P. (1975) Ann. New York Acad. Sci. 253, 60–77.
[10] Kirshner, M. W., Honig, L. S. and Williams, R. C. (1975) J. Mol. Biol. 99, 263–276.
[11] Oosawa, F. (1970) J. Theor. Biol. 27, 69–86.